AD_____
(Leave blank)

Award Number: **W81XWH-08-1-0048**

TITLE: **Functional Characterization of the Protein Product of the Prostate Carcinoma Gene Fusion TMPRSS2:ERG Using the Proteomic and Microarray Analyses**

PRINCIPAL INVESTIGATOR: **Larisa Litovchick, MD., Ph.D.**

CONTRACTING ORGANIZATION: **Dana-Farber Cancer Institute**
Boston, MA 02115

(

REPORT DATE: **July 2009**

TYPE OF REPORT: **Annual**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

- [X] Approved for public release; distribution unlimited
- [ ] Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31-07-2009	Annual	1 July 2008 – 31 Jun 2009

4. TITLE AND SUBTITLE

Functional Characterization of the Protein Product of the Prostate Carcinoma Gene Fusion TMPRSS2:ERG Using the Proteomic and Microarray Analyses

5a. CONTRACT NUMBER
W81XWH-08-1-0048

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Larisa Litovchick, M.D., Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dana-Farber Cancer Institute
44 Binney Street, Boston,
MA, 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A recently identified *TMPRSS2:ERG* gene fusion is present in more than half of the prostate cancer cases and could contribute to the pathogenesis of CaP. The proposed research during the first year of performance was focused at identification of native protein complexes formed by ERG using large-scale immunoprecipitation and MudPIT proteomic analysis. During the performance period, experiments towards this aim were successfully completed. Conditions for semi-preparative purifications of the ERG-CTAP as well as the endogenous ERG were optimized. Several potential candidate ERG-binding proteins present in one or more of the ERG pull-downs but not in the controls. These candidate proteins will be further validated in the repeat MudPIT analyses and in the reciprocal IP experiments using recombinant epitope-tagged constructs and transient transfection. Completion of these experiments will results in identification of the true ERG-interacting proteins.

15. SUBJECT TERMS
TMPRSS2:ERG, immunoprecipitation, mass-spectroscopy, interaction, MudPIT analysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. Introduction.

Project Title: Functional characterization of the protein product of the prostate carcinoma gene fusion *TMPRSS2:ERG* using the proteomic and microarray analyses.

Background. A recently identified *TMPRSS2:ERG* gene fusion is present in more than half of the prostate cancer cases and could contribute to the pathogenesis of CaP [1]. This genetic fusion results in an increased expression and/or alteration of the amino acid sequence of ERG. This in turn could result in abnormal transcriptional activity of this factor mediated by its specific molecular interactions and binding to DNA. Given the established oncogenic role of ETS factors in other cancers, the mutant ERG or ERGa could contribute to the onset and development of CaP. Identification of the native protein complex formed by an ERGa protein product of *TMPRSS2:ERG* gene fusion as well as its direct target genes will help to identify the critical mechanisms of oncogenicity of this CaP-specific mutation.

In order to determine the mechanisms of the oncogenic activity of the product of ERGa in CaP, we proposed to achieve the following **Specific Aims:**

1. To identify native protein complexes formed by ERGa in CaP cells using large-scale immunoprecipitation followed by MudPIT proteomic analysis.

2. To determine target genes regulated by the ERGa protein complex in CaP cells using chromatin immunoprecipitation (ChIP) and global promoter microarray analysis (ChIP-chip).

3. To determine the role of identified ERGa-associated protein complex as well as of its selected target genes in prostate cell transformation and CaP progression using gain of function (overexpression) and loss of function (RNAi mediated depletion) assays.

Study Design: We will isolate ERGa-associated proteins from CaP cells and identify them using MudPIT analysis [2,3]. We will optimize ChIP conditions for ERGa and its partners and use ChIP-chip analysis with human promoter microarray to determine the direct target genes of this complex. We will use bioinformatic resources to classify ERGa target genes and to pinpoint the factors that could contribute to cancer. Finally, we will use prostate cell transformation assays and shRNA depletion to determine the role of members of the ERGa complex as well as its targets in the development and progression of CaP.

Progress. During the first year of the program, a critical set of reagents was generated and the necessary experimental conditions were optimized. A panel of antibodies was extensively tested in different assays and suitable reagents were selected for the future experiments. Expression constructs were generated and sequenced. Some modifications of the initially proposed experimental approach have been made due to an unexpected technical problem with growing VCaP cells stably expressing an epitope-tagged ERG allele. Additional cell lines to express an epitope-tagged ERG were prepared that were not initially proposed and an alternative approach is being tested to express ERG in VCaP cells. The results and findings obtained during the first year of the program as proposed in the original **Statement of Work** are described below.

Body.

Specific Aim 1. To identify native protein complexes formed by ERGa using large-scale immunoprecipitation from CaP cells followed by MudPIT proteomic analysis.

Task 1. To establish the cell lines to be used for purification and validation of the native protein complexes containing ERGa (Months 1 – 6)

a. Expand and freeze stock vials of VCaP prostate cancer cells obtained from ATCC (Months 1-2).

VCaP cells (ATCC Catalog number CRL-2867) were obtained from ATCC and expanded using the recommended growth medium. Frozen stocks of VCaP cells were prepared and successfully tested for viability. In addition, LNCaP cells that do not express ERG were also purchased, expanded and cryopreserved to use as a negative control in the experiments on characterization of the ERG antibodies.

b. Optimize the androgen (R1881) induction of VCaP cells for expression of endogenous ERGa (Months 2-3).

To approach this task, we first characterized several different anti-ERG antibodies available to us. Two antibodies were obtained from Santa-Cruz Biotech (C20 sc-353 and C17 sc-354). Five pre-marketing antibody samples (Ab5 – 9) were obtained for testing from Bethyl Inc., and one antibody (Ab 10) was custom made for us by New England Peptide, Inc. In addition, several different monoclonal antibodies against human ERG are currently being generated at the DFCI-Harvard Cancer Center's Monoclonal Antibody Core and will become available to us in the future.

We tested anti-ERG antibodies in Western blot assay using LNCaP (negative control) and VCaP cells treated for 3 days with 1 nm R1881 (Fig. 1). Ab6, Ab7, Ab9 and C17 antibodies all specifically recognized a protein band of 50 kDa in VCaP lysates but not in LNCaP samples. C20 antibody (sc-353) failed to detect any protein in our assay and was not used further (data not shown). Antibodies Ab6, Ab7, Ab9 and C17 were all able to detect a 50 kDa protein band absent in the LNCaP cell extract. This protein band most likely corresponds to a form of ERG expressed in VCaP cells resulting from a fusion with TMPRSS2 gene.

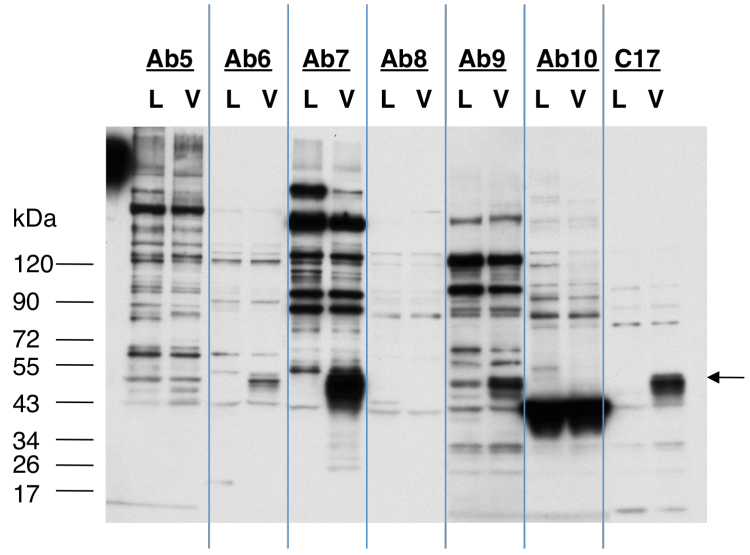

Fig. 1. Western blot screening of an anti-ERG antibody panel.

Lysates were prepared from R1881-induced LNCaP (L) or VCaP (V) cells and 60 mcg of protein was loaded per lane onto a 4-15% gradient SDS-PAGE. The proteins were transferred to a nitrocellulose membrane that was then blocked, cut into strips as indicated and incubated with the specified antibodies (1 mcg/ml) overnight before developing. Position of the molecular weight markers is shown on the side. A specific protein band of 50 kDa is marked by arrow.

Next, we characterized anti-ERG antibody panel in the native immunoprecipitation (IP) assay. As shown in Fig. 2, several antibodies were able to specifically IP a 50 kDa form of ERG from VCaP cells.

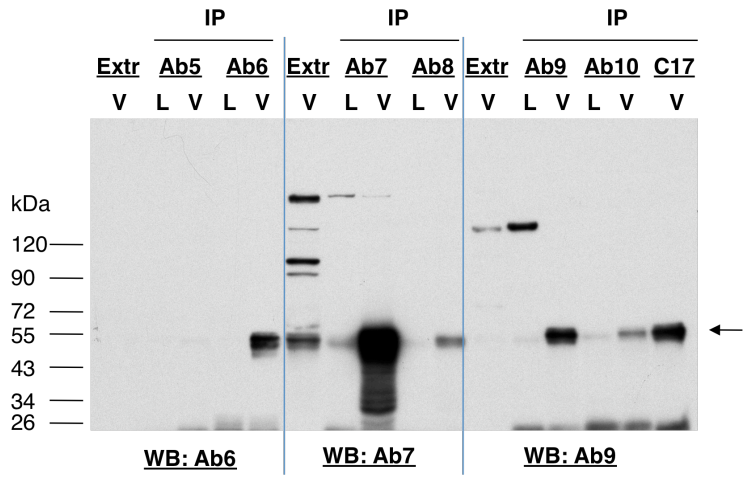

Fig. 2. Native IP screening of the anti-ERG antibody panel. LNCaP (L) or VCaP (V) cells were induced for 3 days with 1nM R1881 and used to prepare cell extracts. Two milligrams of total protein were used for IP with indicated antibodies (2 mcg/ml). The samples were analyzed by SDS-PAGE followed by WB with anti-ERG antibodies as indicated at the bottom of each panel (1 mcg/ml). The blots were developed using Reliablot kit (Bethyl Inc.) to avoid the signal from the IgG heavy chain. The input lanes (Extr) contain 30 mcg of the VCaP (V) cell extract.

To compare the efficiency of the ERG antibodies in the IP of native ERG, we used Ab6, Ab7, Ab9, Ab10 and C17 antibodies to immunoprecipitate ERG from VCaP cells and Western blotted all samples with anti-ERG-Ab7. As shown n Fig. 3, these antibodies were able to IP native ERG with comparable efficiency and could therefore be further tested for their potential use in the large scale IP/mass-spectroscopy and in the chromatin IP.

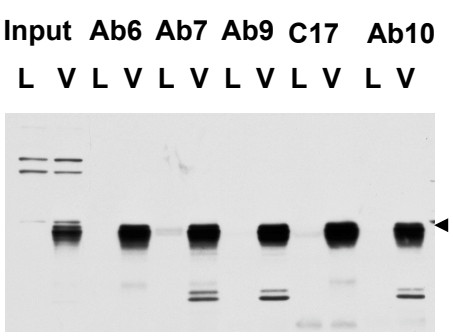

Fig. 3. Characterization of a panel of rabbit polyclonal antibodies that efficiently IP native ERG. LNCaP (L) or VCaP (V) cells were induced with R1881 as above and 2 milligrams of total protein were used for IP of ERG as indicated. The samples were analyzed by SDS-PAGE followed by WB with anti-ERG Ab7 (1 mcg/ml). The signal was detected using Reliablot kit (Bethyl Inc.). The input lanes contain 30 mcg of the LNCaP (L) or VCaP (V) cell extracts. The specific protein band of 50 kDa marked by arrow was detected in the VCaP input lane and in the IP lanes with anti-ERG antibodies.

Milestone: Five different anti-ERG antibodies were found to be positive in the IP of endogenous ERG from VCaP cells. Two antibodies (Ab7 and C17) were also able to specifically recognize the endogenous ERG in WB.

Having selected antibodies for a specific detection of the endogenous ERG using a WB assay, we proceeded to an optimization of the androgen-induced expression of the TMPRSS:ERG fusion product. VCaP cells were grown till sub-confluency and then the fresh growth medium was added, either with 1 nM R1881 or vehicle. The cells were collected for analysis before the change of medium as well as after 24 hours (day 1) or 3, 5 and 7 days of incubation. As shown in Fig. 4, treatment of the VCaP cells with R1881 resulted in up-regulation of EGR expression that was sustained for 7 days. Addition of a fresh growth medium also resulted in a modest up-

regulation of EGR protein levels, possibly due to a presence of the low levels of the steroid hormones in the fetal bovine serum added to the medium.

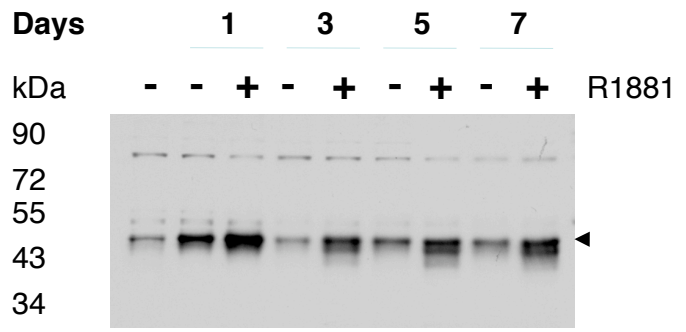

Fig. 4. Induction of ERG expression in VCaP cells.
VCaP cells were treated as described in the text above and used to prepare cell extracts. 30 mcg of protein was loaded per lane and analyzed by SDS-PAGE and WB with anti-ERG Ab7. The sample of the VCaP cells before the treatment was started is loaded in the first lane.

Next, we determined whether the nucleo-cytoplasmic distribution of ERG was affected by induction with R1881. The levels of ERG in the nucleus and cytoplasm were compared in non-induced VCaP cells and after 3 days treatment with R1881. We found that the induced ERG remains predominantly nuclear (Fig. 5).

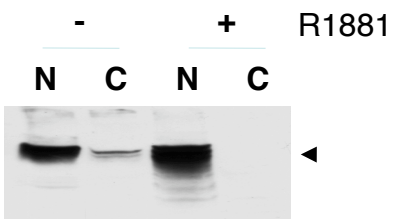

Fig. 5. Induction of ERG expression in VCaP cells results in predominantly nuclear localization of the protein.
VCaP cells were treated as described in the text above and used to prepare the nuclear (N) and cytoplasmic (C) extracts using NePer fractionation kit (Thermo-Fisher). Normalized protein lysates were analyzed by SDS-PAGE and WB with anti-ERG Ab7.

c. Isolate RNA from VCaP cells and clone ERGa by RT-PCR into pBabe-Puro vector with HA or Flag epitope tags to generate ERGa-HA and ERGa-Flag; prepare corresponding retroviruses (Months 1-3)

VCaP cells reportedly express multiple splice variants of EGR mRNA (REF) therefore we decided to use a full-length un-spliced form for preparing the epitope-tagged ERG constructs. The cDNA clone encoding for ERG was obtained from DFCI ORFeome collection and subcloned into the pMSC-Puro-CTAP retroviral vector in frame with a tandem HA-Flag epitope using the Gateway™ cloning system (Invitrogen). The resulting ERG-CTAP construct was further mutated using the QuickChange™ mutagenesis kit (Agilent) to generate the ERGa-CTAP construct lacking the N-terminal 39 amino acids. The constructs were sequenced and transiently expressed to confirm the expression of the polypeptides of the expected molecular weight.

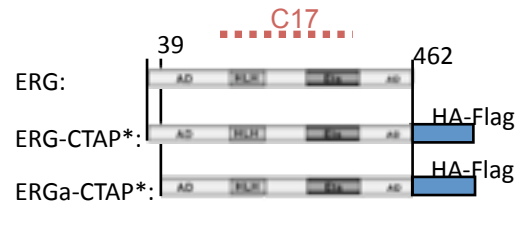

Fig. 6. Schematic presentation of the epitope-tagged ERG expression constructs.
Numbers indicate positions of amino acids and boxes show the functional domains of ERG. HA-Flag tag at the C-terminus is shown as a blue box. Dashed line shows an approximate position of the C17 antibody epitope.

d. Generate VCaP/ ERG-HA, VCaP/ERGa-Flag and VCaP/Babe stable cell lines (Months 3-6)

The retroviruses obtained in Task 2.c. were used to prepare stable cell lines using VCaP cells. We used ERG-CTAP, ERGa-CTAP and vector-CTAP retroviruses to infect VCaP cells. After several days of antibiotic selection using puromycin, we observed a stable increase in cell number in the vector-infected VCaP cells but not in the ERG- or ERGa- infected VCaP cells that apparently failed to proliferate. We then repeated the infection of the VCaP cells together with two other non-prostate cancer human cell lines, T98G and U2OS, in order to test for cell-line specific effects. After infecting equal number of cells with the viruses carrying either empty vector, ERG-CTAP or ERGa-CTAP, antibiotic selection was applied for 1 week after which the cell were collected and counted using the Guava™ FACS analyzer with the ViaCount™ kit (Millipore). As shown in Fig. 7, VCaP cells infected with ERG or ERGa once again failed to proliferate while two other cell lines were unaffected by ERG expression. We were able to generate and to maintain T98G, U2OS and 293T stable cell lines that were all expressing comparable levels of the recombinant ERG or ERGa. No expandable stable clones emerged from the VCaP cells infected with ERG retroviruses even after a prolonged culturing for up to three weeks. It appears that VCaP cell proliferation is specifically inhibited by introduction of the ectopically expressed ERG forms. This interesting observation could indicate that the CTAP-tagged ERG might have a dominant-negative effect in VCaP cells and warrants a further investigation.

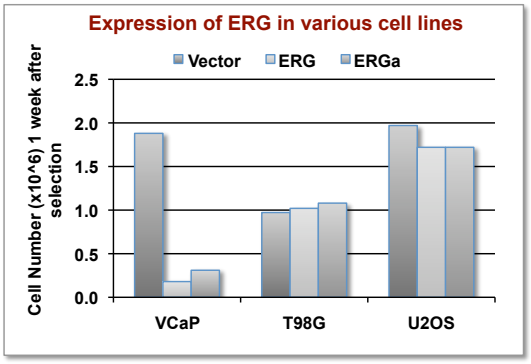

Fig. 7. ERG-CTAP constructs have a growth inhibitory effect in VCaP but not in some other cell types.

The three cell lines were seeded in triplicates at the density of $0.5x10^5$/ml in 6-well plates and infected for 24 h with the same batch of the retroviruses carrying either empty vector, ERG-CTAP or ERGa-CTAP. The cells were then incubated in the growth medium containing 1 mcg/ml puromycin for 1 week. The T98G and U2OS series were split 1:5 at day three because of their relatively faster growth. All cell lines were collected at day 8 and the cell numbers in each series were determined using the Guava ViaCount™ kit. Average values of the triplicate samples are shown.

Milestone: Stable cell lines for expression of ERG-CTAP ad ERGa-CTAP were generated using human T98G, U2OS and 293T cell lines. Ectopic expression of these proteins in VCaP cells resulted in the unexpected and specific failure of these cells to proliferate that made the generation of the proposed VCaP/ ERG-CTAP and VCaP/ERGa-CTAP stable cell lines impossible. Since the VCaP cells still appear to be an optimal and specific model to study ERG, the following steps are being taken to overcome this problem: 1) Cloning and expression of the N-terminally tagged ERG proteins that could have a different effect on VCaP cell proliferation; 2) Since the effect of ectopic ERG in VCaP cells could involve specific protein interactions of physiological significance, an inducible expression system that allows a controlled expression of ectopic ERG in VCaP cells, is being generated; 3) Purification of the endogenous ERG from VCaP cells is being optimized.

e. Verify the expression of ERGa-HA and ERGa-Flag proteins in the generated stable cell lines (Months 4-6)

To optimize the purification of ERGa-CTAP from human cells, we prepared stable T98G and 293T cell lines expressing this protein. Fig. 8 shows that T98G-ERGa-CTAP and 293T-ERGa-CTAP express comparable levels of the target protein but no endogenous ERG. Ectopically expressed ERG could be efficiently immunoprecipitated from these cells using anti-HA, Flag or endogenous ERG antibody.

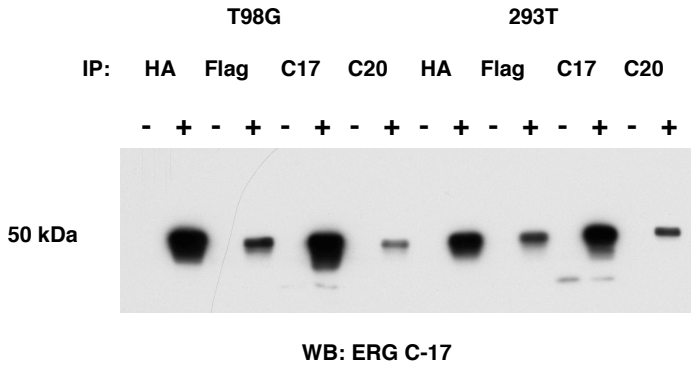

Fig. 8. Expression of ERGa-CTAP in T98G and 293T cells.

Parental cells were infected with a retrovirus carrying ERG-CTAP and selected on puromycin. Expression of ERG-CTAP was tested in the IP-Western blot experiment with indicated antibodies. ERG-C17 and C20 antibodies were from Santa-Cruz Biotech.

Task 2. To optimize the conditions for native and epitope-tag affinity purification of the TMPRSS2-ERG fusion product from CaP cells (Months 6 – 9).

a. *Perform semi-preparative purifications of ERGa-HA using anti-HA IP and ERGA-Flag using anti-Flag IP from VCaP cell lines prepared in Task 1 (Month 6-9)*

We were unable to generate VCaP-ERGa-CTAP cell line due to a growth suppressing effect of the EGR construct. Therefore, we performed a series of semi-preparative IP experiments using T98G-ERG-CTAP and 293T-ERG-CTAP cells instead. Fig. 9 shows an example of experiment using T98G cells as a source to purify ERG-CTAP and ERGa-CTAP using HA pull-down. A similar experiment was performed with 293T cells yielding comparable results (data not shown). Immunoprecipitation using anti-FLAG antibodies reproducibly resulted in less efficient purification of ERG-CTAP and in an inferior quality of the sample (data not shown).

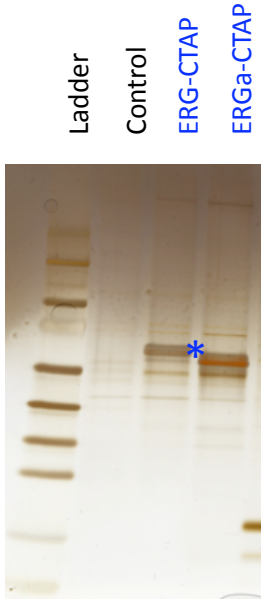

Fig. 9. Purification of ERG-CTAP and ERGa-CTAP from T98G cells using HA pull-down.

T98G-vector, T98G-ERG-CTAP and ERGa-CTAP cells were grown on three 15-cm dishes and collected by scraping the cells into a cold PBS buffer. Cells were pelleted and the cell extracts were prepared by lysis in 5 ml of the IP buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP40 and a cocktail of proteinase inhibitors (Calbiochem). Extracts were centrifuged at 30,000xg for 30 min and used for IP with anti-HA antibody (Sigma). After incubation with anti-HA antibodies, the beads were extensively washed with high-salt IP buffer containing 300 mM NaCl. The complexes were then eluted from the beads using HA peptide (Sigma). The eluates were resolved in 4-15% Criterion™ gradient gel (Bio-Rad) and silver-stained using the ProteoSilver™ kit (Sigma). Position of the molecular weight standards (17 to 160 kDa range) is shown on the left. Asterisk shows a position of the target protein bands.

Next, we tested a panel of anti-ERG antibodies in the semi-large scale IP of native ERG from prostate cancer cells. Several commercial antibodies performed reasonably well in the IP-Western of ERG from human cells and our further testing identified anti-ERG Ab7 as the best antibody to pull down an endogenous ERG. As shown in Fig. 10, Ab7 immunoprecipitated ERG from VCaP cells induced with R1881 for 3 days but not from control LNCaP cells. Incubation of the immunoprecipitates with Ab7-blocking peptide resulted in the elution of the fraction of the immunoprecipitated ERG from the beads. The peptide elution of ERG from the beads will be further optimized to increase the yield of the purified ERG complexes.

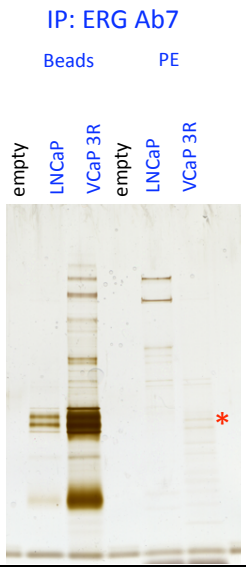

Fig. 10. Purification of native ERG from VCaP cells.

LNCaP (a negative control) and VCaP cells were plated onto three 15-cm dishes at 50% confluency and induced for 3 days with 1 nM R1881. The cells were then collected by scraping off the plate. Extracts were prepared by lysis in 5 ml of the IP buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP40 and a cocktail of proteinase inhibitors (Calbiochem). Extracts were centrifuged at 30,000xg for 30 min and used for IP with anti-ERG Ab7. After incubation with anti-ERG antibodies, the beads were extensively washed with high-salt IP buffer containing 300 mM NaCl. The complexes were then eluted from the beads using Ab7 blocking peptide. The eluates and the beads after elution were treated with SDS-PAGE loading buffer and the samples were resolved using 4-15% Criterion™ gradient gel (Bio-Rad) before silver-staining using the ProteoSilver™ kit (Sigma). . Asterisk shows a position of the target protein bands in the peptide eluates.

b. If none of the commercial anti-ERGa antibody is suitable, generate new anti-ERGa antibody (Months 6-9).

A new anti-ERG antibody was generated (Ab10) and characterized. This antibody is able to IP native ERG but was less efficient then Ab7 that was developed later. Ab10 will be used in future experiments to validate candidate ERG interacting proteins as well as for the ChIP analysis of ERG binding sites.

<u>Milestone:</u> Conditions for semi-preparative purifications of the ERG-CTAP as well as the endogenous ERG were optimized. Anti-HA pull-down of the ERG-CTAP and Ab7 IP of the endogenous ERG were found most satisfactory for large-scale purifications of the ERG complexes. These IP conditions will be used for triplicate pull-down experiments to identify ERG-interacting proteins.

Task 3. To identify ERGa-interacting proteins by large-scale IP and MudPIT analysis (Months 7 – 15).

a. Perform large-scale purifications of ERGa-HA using anti-HA IP from VCaP/ERGa-HA and VCaP/Babe cells (control) cells; submit triplicate samples for MudPIT analysis (Month 7-10)

Since it was not possible to stably express ERG-CTAP and ERGa-CTAP in VCaP cells, we purified an epitope-tagged ERG and ERGa from 293T and T98G cell lines stably expressing these proteins. Use of two different cell lines is beneficial to eliminate any cell-type specific artifacts and to increase the chance of finding the potential interactors. We performed one large scale IP experiment followed by MudPIT analysis for each of the following three conditions: 1) Flag pull-down from 293T-ERG-CTAP and 293T-vector control cells; 2) Flag pull-down from

T98G-ERG-CTAP, ERGa-CTAP and T98G-vector control cells; 3) HA pull-down from T98G-ERG-CTAP, ERGa-CTAP and T98G-vector control cells. The indicated cell lines were grown till sub-confluent, scraped off the plates and extracted using IP buffer. Extracts were clarified by centrifugation as described above and approximately 200 mg of total cell protein extracts were used for each IP. Table 1 shows a summary of the MudPIT analysis of the identification of ERG peptides (ERG Spectral Counts) as well as all peptides (Total Spectral Counts) in each of the experiments described above. These parameters allow us to estimate the efficiency of the pull-down of the target protein as well as the presence of contaminants and non-specific binding proteins (by comparison of the Total Spectral Counts in the bait and the control pull-down). In addition, a Normalized Distributed Spectral Abundance Factor is calculated for the bait protein and reflects a relative enrichment of this protein in the IP sample. As shown in Table 1, ERG was detected in all pull-down experiment but with significantly different abundance. Based on the number of ERG spectra detected and on the ERG enrichment factor dNSAF, the HA pull-down of ERG-CTAP proteins was superior when compared to Flag pull-down. After filtering out the proteins present in the control pull-down sample and several common contaminants (keratins, albumins, mitochondrial proteins), we identified 96 proteins that were present in both HA-ERG and HA-ERGa pull-downs but not in the control IP (data not shown). These proteins are likely to include potential ERG-interacting proteins and will be further validated by repeat MudPIT experiments using HA antibody pull-down.

Table 1. Summary of the epitope-tag pull-down MudPIT analyses of ERG-CTAP and ERGa-CTAP.

	ERG-CTAP-293T Flag-IP PE	293T-Vector Flag-IP PE	ERG-CTAP-T98G Flag-IP PE-SDS	ERGa-CTAP-T98G Flag-IP PE-SDS	T98G-vector Flag-IP PE-SDS	ERG-CTAP-T98G HA-IP PE	ERGa-CTAP-T98G HA-IP PE	T98G-vector HA-IP PE
ERG SC	**19**	**0**	**56**	**282**	**0**	**997**	**1174**	**0**
Total SC	353	1513	1259	5387	6240	4749	4251	2889
Total Proteins	**81**	**106**	**127**	**191**	**211**	**271**	**337**	**173**
ERG dNSAF	0.03	0.00	0.02	0.03	0.00	0.20	0.26	0.00
FDR (%)	**0.565**	**0.9211**	**0**	**0.2595**	**0.1921**	**0.0842**	**0.2819**	**0**

Table shows a summary of the pilot epitope-tag ERG pull down experiments. Gray columns indicate the negative control experiments. ERG SC – number of spectral counts including non-unique peptides derived from ERG sequence; Total SC – number of spectral counts from all identified proteins; Total proteins – a number of unique proteins identified; ERG dNSAF - Normalized Distributed Spectral Abundance Factor for ERG; FDR – false discovery rate.

b. Perform large-scale purifications of endogenous ERGa from R1881-stimulated and control VCaP cells using anti-ERG antibodies; submit triplicate samples for MudPIT analysis (Months 10-13)

Next, the endogenous ERG pull-down conditions were tested using ERG Ab7 and Ab17 and either native ERG from VCaP cells or ectopically expressed ERG-CTAP in T98G cells. Analysis of the endogenous ERG pull-down form VCaP cells induced with R1881 for 3 days resulted in detection of 133 spectral counts of ERG-derived peptides and identification of 8 different proteins. A control LNCaP cell pull-down only resulted in identification of 1 protein represented by 6 spectral counts. Both of these samples resulted in identification of an atypically low number of Total Spectral Counts. It is not clear whether this result is due to a technical issue with mass-spectroscopy analysis or due to a quality of the immunoprecipitated sample. As in the experiment shown in Fig. 10, elution with the Ab7 blocking peptide resulted in recovery of only a small fraction of the target protein. This step is currently being optimized further and it is anticipated that with improved elution, MudPIT analysis of the Ab7 pull-down from VCaP cells

will produce valuable information about the endogenous ERG complexes. Alternatively, we will use a different antibody that is able to IP native ERG such as Ab6, 9 or 10 for the analysis of the endogenous ERG complexes from VCaP cells.

Table 2. Summary of the native pull-down MudPIT analyses of ERG.

	VCaP Ab7-IP PE	LNCaP Ab7-IP PE	VCaP C17-IP PE	ERG-CTAP-T98G C17-IP PE	T98G-vector C17-IP PE
ERG SC	**133**	**0**	**143**	**111**	**0**
Total SC	360	6	3008	3214	3087
Total Proteins	**8**	**1**	**107**	**138**	**121**
ERG dNSAF	0.31	0.00	0.04	0.03	0.00
FDR (%)	**4.3478**	**80**	**0.2656**	**0.4346**	**0**

Table shows a summary of the pilot native ERG pull down experiments. Gray columns indicate the negative control experiments. ERG SC – number of spectral counts including non-unique peptides derived from ERG sequence; Total SC – number of spectral counts from all identified proteins; Total proteins – a number of unique proteins identified; ERG dNSAF - Normalized Distributed Spectral Abundance Factor for ERG; FDR – false discovery rate.

We then used the T98G-ERG-CTAP expression system to test another ERG antibody, C17 (Santa-Cruz Biotech) and to compare the recombinant ERG pull-down with the endogenous ERG from VCaP cells. This antibody was also found to efficiently IP ERG in the preliminary testing and the blocking peptide for elution from the beads was available from Santa-Cruz Biotech as a custom order. MudPIT analysis of the C17 pull-downs from R1881-induced VCaP cells as well as from T98G-ERG-CTAP cells, resulted in efficient pull-down and elution of ERG as shown in Table 2. However, C17 pull-downs also contained very high levels of contaminants that interfered with the overall quality of the analysis (reflected in a low ERG dNSAF value, Table 2). Analysis of the C17 pull-downs from T98G cells and from VCaP cells resulted in identification of 37 proteins that appeared specific, i.e. were absent in the control IP and did not belong to a list of common contaminants (data not shown). From this list, 9 proteins were also present in the HA pull-downs from T98G-ERG-CTAP or ERGa-CTAP cells (data not shown).

c. If no suitable antibody for endogenous ERGa purification is selected (Task 2), perform large-scale purifications of ERGa-Flag using anti-Flag IP from VCaP/ERGa-Flag cells and VCaP/Babe cells: submit triplicate samples for MudPIT analysis (Month 10-13)

The purification of the endogenous ERG seems to produce promising results in a pilot experiment described above. It is anticipated that the two more repeats of the MudPIT analysis using Ab7 and C17 IP together with the HA IP of the ERG-CTAP will result in identification of specific ERG-interacting proteins of high confidence.

d. <u>Milestone:</u> Analyze molecular components of the isolated complexes and identify specific binding proteins present in ERGa complexes purified using two different antibodies and absent in control IPs (Months 10-15)

We identified several potential candidate ERG-binding proteins that were present in one or more of the ERG pull-downs but not in the controls. These candidate proteins will be further validated in the repeat MudPIT analyses and in the reciprocal IP experiments using recombinant epitope-tagged constructs and transient transfection. Completion of this step in the next few months will results in identification of the true ERG-interacting proteins.

Key Research Accomplishments

- **Several commercially available and newly made antibodies against human ERG were extensively characterized. Five different antibodies suitable for native immunoprecipitation (IP) of human ERG were selected.**

- **DNA constructs for preparing stable cell lines expressing the carboxyl-terminally fused with HA-Flag tandem epitope tag ERG and ERGa alleles (ERG-CTAP and ERGa-CTAP) were generated.**

- **Upon attempting to generate VCaP (ERG-positive) prostate cancer cell lines expressing ERG-CTAP and ERGa-CTAP, observed an unexpected and robust growth suppression effect of the ectopically expressed ERG alleles.**

- **This growth suppression effect was found to be cell-type specific since the proliferation of three other human non-prostate cancer cell lines 293T, T98G and U2OS was not inhibited by the ectopic expression of ERG-CTAP or ERGa-CTAP.**

- **293T-ERG(a)-CTAP, T98G-ERG(a)-CTAP and U2OS-ERG(a)-CTAP stable cell lines were produced to use for purification of the epitope-tagged ERG and for validation of the candidate ERG-interacting proteins.**

- **Conditions for the endogenous and the epitope-tagged IP of human ERG for mass-spectroscopy analysis were optimized and suitable antibodies were selected.**

- **Several experimental conditions for mass-spectroscopy analysis of ERG complexes were tested in a large-scale IP followed by MudPIT proteomic analysis.**

- **Based on the MudPIT analysis data, the best antibodies for ERG IP were selected and the optimal conditions for the further ERG purifications were determined.**

- **Preliminary list of candidate ERG-interacting proteins was generated.**

Reportable Outcomes

Presentations: March 2009, Baltimore, Maryland: Annual Prostate Cancer Program Retreat organized by Dana-Farber Cancer Institute, Memorial Sloan Kettering Cancer Center, Johns Hopkins School of Medicine, and the University of Michigan.

Submitted an abstract and presented a short talk entitled "**Functional Characterization of the Protein Encoded by TMPRSS2:ERG fusion".**

Reagents: Several antibodies were extensively characterized that are able to specifically recognize human ERG in the native immunoprecipitation assay. One of these anti-ERG antibodies was custom made specifically for this project. Expression constructs for ERG and ERGa were generated to express HA-Flag (CTAP) tagged alleles.

Development of cell lines: Stable cell lines expressing ERG-CTAP, ERGa-CTAP or vector were generated using retroviral transfer and antibiotic selection. Human 293T, U2OS and T98G cells were used as parental cell lines to infect with retroviruses. In total, nine cell lines were generated for this project. The originally proposed VCaP cell line failed to proliferate upon the infection with ERG-expressing viruses due to specific biological effect of ectopically expressed ERG in these cells. The proposed VCaP-ERG-CTAP cell line was not generated because of this reason. Growth suppressing effect of ERG-CTAP in VCaP cells need to be further investigated.

Employment opportunities applied for: Several applications for a faculty position were submitted during the year to various research centers with strong and established prostate cancer research programs.

Conclusion

This research effort is aimed at the molecular characterization of the important new proto-oncogene that is implicated in prostate cancer and results from a genetic fusion of the TMRSS2 and ERG genes. During the first year of the program, a critical set of reagents was generated and the necessary experimental conditions were optimized to detect, isolate and biochemically characterize the protein product of the TMPRSS:ERG genetic fusion. A panel of critical reagents was generated. Several new antibodies was extensively tested in different assays and suitable reagents were selected for the future experiments. Expression constructs were generated and sequenced.

An unexpected problem was encountered with growing VCaP cells stably expressing an epitope-tagged ERG allele. Interestingly, the potent growth-suppressing effect of the ectopic expression of ERG was VCaP cell-specific and was not observed in three different non-prostate cancer cell lines. It is possible that the epitope-tagged ERG acts in a dominant-negative way in VCaP cells. This interesting phenomenon was not previously reported and warrants a further investigation. Additional cell lines to express an epitope-tagged ERG were prepared that were not initially proposed and an alternative approach is being tested to express ERG in VCaP cells in an inducible fashion.

Significant progress has been made in identification of the ERG interacting proteins. We were able to use anti-ERG antibodies for purification of the endogenous ERG from VCaP cells and epitope tag antibodies to purify the ectopically expressed ERG from two different non-prostate cancer cell lines. Purification conditions have been optimized and the best performing antibodies have been identified. In several pilot mass-spectroscopy experiments using both native and ectopically expressed ERG, a list of potential interacting proteins was generated that will be further validated.

References

1. Demichelis F, Rubin MA: TMPRSS2-ETS fusion prostate cancer: biological and clinical implications. *J Clin Pathol* 2007, 60:1185-1186.
2. Florens L, Washburn MP: Proteomic analysis by multidimensional protein identification technology. *Methods Mol Biol* 2006, 328:159-175.
3. Litovchick L, Sadasivam S, Florence L, Zhu X, Swanson SK, Velmurugan S, Washburn MP, Liu XS, DeCaprio JA: Evolutionally conserved multi-subunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. *Molecular Cell* 2007, 26.